J. Dairy Sci. 99:1–12
http://dx.doi.org/10.3168/jds.2016-11167

Investigating bacterial population structure and dynamics in traditional koumiss from Inner Mongolia using single molecule real-time sequencing

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ABSTRACT

Koumiss is considered as a complete dairy product high in nutrients and with medicinal properties. The bacterial communities involved in production of koumiss play a crucial role in the fermentation cycle. To reveal bacterial biodiversity in koumiss and the dynamics of succession in bacterial populations during fermentation, 22 samples were collected from 5 sampling sites and the full length of the 16S ribosomal RNA genes sequenced using single molecule real-time sequencing technology. One hundred forty-eight species were identified from 82 bacterial genera and 8 phyla. These results suggested that the structural difference in the bacterial community could be attributed to geographical location. The most significant difference in bacterial composition occurred in samples from group D compared with other groups. The sampling location of group D was distant from the city and maintained the primitive local nomadic life. The dynamics of succession in bacterial communities showed that *Lactobacillus helveticus* increased in abundance from 0 to 9 h and reached its peak at 9 h and then decreased. In contrast, *Enterococcus faecalis*, *Enterococcus durans*, and *Enterococcus casseliflavus* increased gradually throughout the fermentation process, and reached a maximum after 24 h.
Keywords: koumiss, biodiversity, predominant species, SMRT sequencing, fermentation succession

INTRODUCTION

Koumiss (kumis), also called airag, arrag, and chige in the Mongolian language, is a fermented mare milk beverage that is popular in Central Asia and Eastern Europe. The production of the ancient beverage koumiss can be traced to the ancient Greeks and Romans in Europe (Wszolek et al., 2006). In China, koumiss also has a long history as a popular drink among traditional nomadic people. The earliest record of koumiss appeared during the Han dynasty (202 BC–202 AD) and its popularity became widespread during the Yuan dynasty (1271 AD–1368 AD; Zhang and Zhang, 2012). Koumiss has long been considered as a complete food, rich in nutrients with medicinal properties. In the 18th century, koumiss was thought to have therapeutic value in the treatments of wasting diseases caused by phthisis, abdominal tumors, and dyspepsia (Thompson and Leamnington, 1879). In recent years the Cossacks have introduced koumiss into military rations to prevent tuberculosis (Ishii and Samejima, 2001). Chen et al. (2010) found that koumiss is rich in angiotensin I-converting enzyme inhibitory peptides, which have antihypertensive properties. Chaves-López et al. (2012) also reported angiotensin I-converting enzyme inhibitory activity in yeast strains isolated from Colombian koumiss.

Traditionally, fermentation of koumiss was achieved in wooden casks or in bags made of animal skin (Zhang and Zhang, 2012). At present, porcelain urns may also be used. In general, koumiss is produced by fermentation of natural microflora, including lactic acid bacteria (**LAB**) and yeasts, at ambient temperatures (approximately 20°C) for 1 to 3 d (Park et al., 2006; Sun et al., 2010). During koumiss manufacture, filtered fresh mare milk is poured into urns that are placed outside the Mongolian yurt, and beaten or stirred with a wooden stick to ensure mixing evenly and fast fermentation. Every day, after removing what is required for consumption, a small aliquot of koumiss is retained for use as a starter culture for the next day, and more milk is added to ensure ongoing fermentation.

The microorganisms responsible for fermentation of koumiss mainly consist of LAB and yeast. Due to the therapeutic effects of koumiss and the fact that it is regarded as a valuable natural source of LAB, interest has been growing in identifying the microflora in koumiss by culture-dependent and culture-independent

Received March 14, 2016.
Accepted July 3, 2016.
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Table 1. Samples of koumiss that had been fermented for different lengths of time and collected from different sites in Xinlingol

Sample	Site 1	Site 2	Site 3	Sites 4	Site 5
Fresh mare milk	A1[1]	B1	C1[1]	D1	E1[1]
0 h	A2[1]	B2	C2[1]	D2[1]	E2
3 h	A3	B3	C3	D3	E3
6 h	A4	B4	C4	D4	E4
9 h	A5	B5	C5	D5	E5
12 h	A6	B6[1]	C6	D6[1]	E6[1]
>24 h	—	B7	—	—	—

[1]DNA was not successfully extracted, so sequencing was not possible for these samples.

methods. Much research has been done to identify the LAB community using pure culture methods and has suggested that the predominant LAB in koumiss is *Lactobacillus* and *Lactococcus* species (Oberman and Libudzisz, 1985). An et al. (2004) also found that the isolates in koumiss from Inner Mongolia were mainly *Lactobacillus* species. Sun et al. (2010) systematically investigated the *Lactobacillus* species in homemade koumiss in Xinjiang, Inner Mongolia, and Qinghai by the combined use of conventional and molecular methods. The overall distribution pattern demonstrated that the *Lactobacillus* groups from the 3 geographically distant regions varied significantly. Hao et al. (2010) studied bacterial biodiversity in traditional koumiss by denaturing gradient gel electrophoresis with similar results. However, the ratios of the most frequently isolated species varied between sampling sites. Culture-dependent methods are commonly used, but it is well recognized that these methods often fail to characterize (minor) populations of microorganisms that require selective enrichment. Furthermore, conventional methods are not able to detect noncultural species, which represents a serious limitation for identification of all species present in any given sample.

Pyrosequencing-based 16S ribosomal RNA (**rRNA**) profiling has emerged as a powerful technique to characterize the structure of microbial communities in ecological samples (Justé et al., 2008; Humblot and Guyot, 2009; Desai et al., 2010; Faveri et al., 2015). The single molecule real-time sequencing technology (**SMRT**) of Pacific Biosciences (Menlo Park, CA) is one of several next-generation sequencing technologies that are also currently in use. This new technology not only produces considerably longer and more accurate DNA sequences from individual unamplified molecules, but it is also as reliable and accurate as more traditional approaches (Bashir et al., 2012; Roberts et al., 2013). It can quickly and accurately identify bacteria to the species level. To understand the structure and population dynamics of bacterial communities in koumiss we used SMRT sequencing to characterize the species present in 22 samples of traditional koumiss collected at different times during the fermentation process, from 5 sampling sites in Xinlingol, Inner Mongolia.

MATERIALS AND METHODS

Sample Collection

A total of 31 traditional fermented koumiss samples were collected from 5 different sites in Xilingol, Inner Mongolia. The 5 sites involved were the places near the city (site 2) and far away from the city (site 4), as well as the family maintaining the original koumiss-making process (sites 1 and 5) and the small workshop-style production koumiss family (site 3). From the site where the family maintains the traditional making process, samples at different stages of fermentation were taken: fresh milk and 0, 3, 6, 9, 12, and >24 h of fermentation (Table 1). Samples were collected aseptically and were transported in a tank of liquid nitrogen. Among these, 9 samples were not evaluated further because we were unable to extract the DNA successfully for subsequent sequencing.

DNA Extraction and PCR Amplification

Genomic DNA was extracted from each sample using Qiagen DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of extracted DNA was checked by 0.8% agarose gel electrophoresis and spectrophotometry (optical density at 260 nm/280 nm ratio). All extracted DNA samples were stored at −20°C before subsequent analysis.

Bacterial 16S rRNA gene was amplified by PCR for bar-coded pyrosequencing. The full-length sequences of the 16S rRNA gene of all bacteria were amplified using the universal forward 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse 1541R (5′-AAGGAGGTGATCCAGCCGCA-3′) primers (Devereux and Wilkinson, 2004). The PCR program was as follows: 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and